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Experiments completed in the past year were designed to characterize the modifications in mammary gland and tumor vascular endothelium. Studies included analysis of VEGF isoform expression and vascular morphology in mammary gland tumors, mammary glands from ovariectomized (implanted with estrogen or placebo pellets), pregnant, lactating and involuting mice. Our findings demonstrate that estrogen alone can up-regulate the expression of VEGF 120, 164, and 188 isoforms and induce continuous vascular endothelium to become fenestrated. In all cases, where estrogen was present, VEGF was up-regulated, vessels were proliferating, vascular endothelium was fenestrateed and caveolae were clustered and fused. Moreover, in situations, where estrogen was lacking or decreasing, vascular endothelium had no fenestrations or clustered, fused caveolae. It is known that microvascular permeability is increased with the presence of fenestrae and clustered, fused caveolae. We hypothesize that estrogen is a physiological mediator of VEGF which results in a regulated increase in microvascular permeability at times of mammary gland vascular proliferation.

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PI - Signature 8/28/98
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(5) INTRODUCTION

Significant progress has been made in the detection and treatment of breast cancer in the past 10 years, however, breast cancer is still one of the leading causes of death in women [1,2]. There are 150,000 new breast cancer diagnoses each year [3]. Surgery alone results in cure or long-term remission in only 50% of the cases and disease recurrence is an astounding 80% within 10 years in node-positive cases [4]. Presently, many tumor characteristics are used as indicators of the future clinical course of women with breast cancer, however, structural or functional components of tumor growth that are associated with aggressiveness are yet unknown [5]. Obviously, more sensitive methods for early detection and a better understanding of the etiology and pathogenesis of breast cancer are necessary to combat this disease.

Tumor growth and metastatic spread of the primary tumor are generally accepted to be angiogenesis dependent. The ingrowth of the surrounding vasculature to supply the tumor with nutrients and provide a conduit in which tumor cells can travel to distant sites is absolutely necessary for tumor growth beyond 2 mm in diameter and for the development of distant metastases [6-12]. Numerous retrospective and prospective studies have demonstrated that angiogenesis (high microvessel density) in clinical breast cancer is positively correlated with increased recurrence, metastasis, and mortality in both node positive and node negative patients and has proven to be one of the best prognostic indicators presently available [13-15].

The mechanisms by which the host vasculature invades tumors are numerous and complicated. However, the primary and critical event is the release of an angiogenic factor from the tumor cells or the surrounding extracellular matrix [16,17]. The search for tumor-derived angiogenic growth factors has been an area of intense effort over the past 20 years. Many angiogenic factors have been described, but only one known factor, vascular endothelial growth factor (VEGF, also known as vascular permeability factor, VPF)[18,19], acceptably satisfies the many criteria to be considered an angiogenic factor outside of experimental models. Originally identified as a tumor-secreted protein which increased vascular permeability *in vivo*, VEGF is also angiogenic *in vivo* [20-22]. VEGF is a homodimeric protein of ~46 kD produced by many cell types, including a variety of tumors,

folliculostellate cells, macrophages, and possibly podocytes or capsular epithelial cells in the renal glomeruli among others [23]. VEGF is highly conserved with 88-98% homology among murine, rat, bovine, and human sequences [24-26]. Interestingly, the VEGF mRNA is alternatively spliced into 4 isoforms of 121, 165, 189, and 206 amino acids (120, 164, 188, and 205 aa in mice) [27,28]. Although all 4 isoforms have a signal peptide sequence, only the two shorter proteins are believed to be secreted while the longer forms remain associated to the cell membrane or extracellular matrix. Yet, the 121 aa isoform does not bind heparin well and the 165 aa isoform does [29]. All 4 species can cause endothelial mitogenesis and increase vascular permeability in experimental systems, but functional differences, relative potencies, and tissue distribution *in situ* are not well established. Since there are significant biochemical differences among the 4 isoforms (secretion, heparin binding), it is reasonable to hypothesize that they will have different functions and/or be produced by different cells or in different locations. In fact, there is evidence that a non-soluble form of VEGF is responsible for vascular patterning in quail development, such as $VEGF_{189}$ [30]. Unlike the other putative tumor angiogenesis factors, bFGF and PD-ECGF, VEGF is specific for endothelial cells. It does not increase mitogenesis in any other cell type and its receptor has only been directly localized to endothelial cells [31,32].

There are two high affinity receptors identified for VEGF, fms-like tyrosine kinase (Flt-1) and fetal liver kinase (Flk-1) (both homodimers of 180 and 205 kD, respectively) [33,34]. As their names suggest, both are receptor tyrosine kinases and appear to be exclusively expressed on endothelial cells. Although the receptors are related to the PDGF receptor, they represent a new class of receptor tyrosine kinases since their extracellular domain contains 7 immunoglobulin repeats rather than the standard 5 [33]. The VEGF receptor mRNA has been detected in the endothelium of many tissues but at different levels of expression, with proliferating vascular endothelial cells (due to either pathology or normal development) expressing the highest levels [35,36]. In fact, except for some fenestrated endothelium in the kidney glomerulus and choroid plexus, non-proliferating vascular endothelium does not express detectable levels of either receptor [36]. The receptors are the first proteins expressed on endothelium in development and are critically necessary for animal development as evidenced by the fact that the receptor knockouts are embryonic lethal [37,38]. However, no studies have adequately quantitated the level of receptor

expression in tumor vasculature. As with the 4 isoforms of VEGF, differences in function and *in vivo* distribution between the 2 receptors in normal or cancer tissue are unknown.

Tremendous research interest has developed in VEGF and its receptor, in part, due to their ubiquitous presence at times of vascular proliferation and unique regulation. Both the growth factor and its receptors have been shown to be intimately involved in developmental vasculogenesis, demonstrating spatial and temporal regulation throughout the vascular development of the embryo [39]. Moreover, VEGF is believed to be involved in, if not responsible for, the angiogenesis during wound healing [40] and numerous pathologies, which include diabetic retinopathy [41], rheumatoid arthritis [42], chronic inflammation [43], psoriasis [44], as well as, numerous malignancies [35,45-47], including breast cancer [14,48]. In short, in every case where angiogenesis is a prevalent pathological characteristic, VEGF has been found.

Many markers are currently used as prognostic indicators in breast cancer. The "perfect" prognostic indicator has been described as one which is functionally involved in the generation and progression of the disease [49]. Studies on breast cancer and its progression (pathogenesis) have resulted in the identification of numerous prognostic indicators. Traditional prognostic indicators have included host factors such as age, menopausal status, and nodal involvement. As the mechanisms of cancer progression and tumor growth became elucidated, new prognostic indicators began to focus on general biological attributes of cancer cells, such as proliferation markers, proteases, growth factors, and oncogenes [49,50]. Much of the data on the prognostic value of these markers has been conflicting, but many of these markers have proven to be moderate to poor indicators of either disease free survival or overall survival [49]. Tumor angiogenesis, measured by counting vessels immunohistochemically stained for CD31 or factor VIII antigen, has proven to be an independent and highly significant prognostic indicator in predicting overall survival and relapse-free survival, being as good as or better than other commonly used indicators [51-54]. Unfortunately, these currently used markers are not "perfect". CD31, although highly expressed on endothelium, is found on other cells (e.g. platelets), has no known function in angiogenesis, and is not specific for neovasculature. Additionally, factor VIII antigen is variably expressed on microvasculature, often not identifying smaller vessels. Furthermore, counting immunohistochemically stained microvessels is a qualitative method subject to

observer variations. The development of a quantitative method of a tumor vascular specific marker which could not be biased by individual observers would be superior. It is possible that the VEGF receptors are excellent prognostic indicators of microvascular density.

In the past year we have undertaken studies to characterize the involvement of all four VEGF isoforms and two receptors in breast cancer using PCR. Additionally we have developed a competitive PCR assay to quantitatively measure the expression of both VEGF receptors, Flk and Flt, in breast cancer. Continued experiments will determine whether measurement of the VEGF receptors is representative of the microvascular density and therefore might replace the qualitative and laborious method of counting immunohistochemically stained microvessels.

(6) BODY OF PROPOSAL

Methods

Tissue Isolation

Female mice were ovariectomized (OVX) and implanted with either an Estradiol pellet or placebo pellet (Innovative Technologies Research, Toledo, OH). Murine (MXT-OVEX) breast carcinoma cells were injected into the 3rd mammary fat pad of ovariectomized mice. Once tumors reached ~5-8 mm diameter, the animals were sacrificed, the tumors were removed and processed for microscopy and biochemical analysis. Additionally, normal mammary tissue from ovariectomized animals with estradiol pellets or placebo pellets was harvested.

Animals were also used for harvesting mammary glands at different stages of development; virgin, pregnant, lactating, and involuting.

Flk & Flt cloning

The C-terminal region of murine Flk-1 (aa 1289-1419) and Flt-1 (aa1277-1396) was PCR cloned from murine fetal RT-cDNA. The PCR fragment was cloned in frame into pGEX-KG and pET28 vectors and used for bacterial expression of GST- or His-tag fusion proteins,

respectively. The GST-protein was used to immunize Longhorn chickens and the His-tagged protein was used for affinity purification.

Antibody generation & purification

A hyperimmunization protocol was used for antibody generation. The initial immunization was done with complete Freunds adjuvant while booster immunizations were done after 1, 2, 4, 8, and 12 week with incomplete Freunds adjuvant. The IgY fraction (comparable to the IgG fraction in other animals) was purified as previously described [55,56]. Briefly, yolks were separated from albumin and gently washed in dH2O to remove any adherent albumin. Yolk contents were mixed with 4 x their volume with PBS (pH 7.4), PEG6000 (Sigma, St. Louis, MO) was added to a concentration of 3.5% and the mixture was centrifuged at 5000 x g for 20 min. The supernatant was saved and the pellet was redissolved with PBS and re-extracted with PEG. The supernatant from the both extractions was combined and filtered through cotton gauze. IgY was precipitated from the filtered supernatants by adding PEG to a final concentration of 12% and centrifuging for 25 min at 5000 x g. The IgG was then dissolved in PBS (2.5 x the starting yolk volume) and reprecipitated with 12% PEG. Precipitated IgG was dissolved in PBS (0.25 x the starting volume of yolk), cooled to 0°C on ice and mixed with 50% EtOH (precooled to -20°C) (0.25 x the starting volume of yolk) to dissolve the PEG and precipitate the IgY. After centrifugation, the pelleted IgY is dissolved in PBS (0.25 x the starting volume of yolk) and dialysed overnight against PBS with 0.02% azide. One yolk (~15 ml) generally yielded 4-5 mg of 95% pure IgY as determined by protein assay and silver stained gel.

For affinity purification, the His-tagged fusion protein (Flk-1 or Flt-1) was conjugated to Affigel 10 beads and an affinity column was made for the antibodies. Purified IgY was mixed with the beads, washed with 10 mM Tris-Cl (pH 7.5) and bound antibodies were eluted with 100 mM glycine (pH 3.5) into neutralizing 1 M Tris (pH 8.0).

ELISA

Eluted fractions were tested for antigen specificity using an ELISA plate coated with both Flk-His and Flt-His. Positive fractions were concentrated in centricon 100 spin concentrators. Antibody purity and concentration were determined by PAGE and BCA,

respectively. To further test the specificity of the Ab, ELISA plates were coated with either membrane or cytosol fractions from MS1 endothelial cells. Purified Ab was tested using standard protocols on these plates.

Western blot

Immunoblotting was completed on membrane fractions of murine endothelial cell line, MS1. Briefly, cells were scraped with tris buffer (pH8.0) containing protease inhibitors without detergents. Lysates were passed through an 18G needle 3 times and centrifuged at 1000 xg for 15 min. The resulting postnuclear supernatant was centrifuged at 100,000 xg for 1 hour and the pellet was resupended in tris buffer with detergents. Protein concentration was determined and 50 μg protein/lane were separated on a 6% gel, transferred to nitrocellulose, blocked with 10% FCS and blotted with purified Ab overnight at 4°C. Following washes and incubation with an HRP-conjugated secondary and ECL, the immunoblots were exposed to film for band detection.

RT-PCR of VEGF

To determine the presence of VEGF isoforms, RT-PCR was used. Primers were designed to amplify all isoforms present in either murine or human tissue samples equivalently (upper: 5'-CACCAAAGCCAGCACATA-3'; lower: 5'-CCGCCTTGGCTTGTCACATC-3'). Tumors were removed and frozen immediately on dry ice then homogenized in a Dounce homogenizer (treated with RNase Zap (Ambion, Austin, TX)) and RNA was extracted using the Trizol reagent (Gibco BRL, Gaithersburg, MD). Prior to reverse transcription, RNA was treated with RNase-free DNase (Promega, Madison, WI) to remove any contaminating genomic DNA [57]. DNased RNA was purified using the RNAeasy kit (Qiagen, Chatsworth, CA), quantitated spectrophotometrically and 2 ug was reverse transcribed using Superscript II and random primers according to the manufacturer's instruction (Gibco BRL). PCR reactions were as follows: 1 μl tumor RT-cDNA, 0.4 μM upper and lower primer, 2.5 μl Pfu PCR buffer, 1 μl dNTP, 20 μCi ^{32}P-dCTP, 16 μl H_2O, and 1 μl Pfu using the following method (95°C 5 min; 95°C 30 sec, 58°C 1 min, 72°C 2 min for 30 cycles; 72°C 10 min). Reactions (15 μl) were separated on a 6% polyacrylamide gel made with Tris

borate EDTA buffer, dried and exposed to a phosphorimager screen. Sample bands were quantitated using ImageQuant analysis software (Molecular Dynamics, Sunnyvale, CA).

Electron Microscopy & Vascular Morphometry

Mice were sacrificed, the mammary gland or tumor was removed and immediately placed in 3% formaldehyde. Smaller pieces of tissue were then fixed in glutaraldehyde and processed as previously described for electron microscopy [58]. Thin (50-55 nm) sections were cut (Reichert-Jung Ultracut E; Vienna, Austria), picked up on copper grids, and stained with uranyl acetate and lead citrate prior to examination and photography (Phillips CM10 electron microscope at 80 kV). Morphometric analysis was determined from at least 3 blocks of 3-5 animals. Statistical analysis was determined using StatView 4.5.

Results

Antibody Verification

Membrane/Cytosol ELISA:

Two chickens were used for each receptor immunization. Initial screens using fusion proteins demonstrated that antibodies specifically recognized the receptor fusion protein. Since the VEGF receptors are localized to the membrane of endothelial cells (EC), an EC cytosol/membrane ELISA was used as an initial test of antigen specificity. Although Abs were positive on fusion protein ELISA, only anti-Flk specifically recognized the membrane fraction (Fig. 1). Antibodies to Flt-1 were not sensitive nor specific enough to merit continued testing. The Flk-1 Ab was used for immunoblotting.

Endothelial cell Western blot

Anti-Flk-1 was tested in immunoblotting along with commercially available Ab for Flk-1 from Santa Cruz Laboratories. In a dilution dependent manner, the 72 Ab specifically recognized a band at 205 kDa only in membrane fractions, corresponding to the Flk-1 receptor (Fig. 2). These results suggest that this Ab specifically recognizes Flk-1. The anti-Flk-1 Ab was also used in western blotting of tumor lysates and membrane fractions of tumor lysates. Unfortunately, the blots were not clean enough to provide conclusive identification of

the receptor. This antibody has been used in preliminary studies to stain endothelial cells growing in culture and appears to be useful for immunohistochemistry. These studies are on-going and will be completed in the next 6 months.

Mammary Vascular Morphology and VEGF isoform expression

We have previously demonstrated that mammary tumors grown in the mammary fat pad generate fenestrated vessels regardless of exogenous estrogen. We followed up on these findings to determine whether normal mammary vasculature would be similarly modified in the presence of estrogen in a VEGF-dependent manner. Normal virgin mammary gland vascular endothelium is non-fenestrated and is morphologically indistinguishable from mammary gland vascular endothelium from ovariectomized (OVX) mice implanted with placebo pellets ($-E_2$) having no fenestrations and many non-fused caveolae (Fig. 3C). Contrary to this, mammary glands in OVX mice with estrogen pellets ($+E_2$) have fenestrated endothelium with fused and clustered caveolae (Fig. 3A, B). Furthermore, mammary vascular endothelium from pregnant (Fig. 3D) or lactating (Fig. 3E) animals are fenestrated and have fused, clustered caveolae. These results are summarized in Table 1, which details the percentage of samples from various tissues or tumors (in general) have fenestrated endothelium. We have previously demonstrated that these modifications are the direct result of VEGF activation of endothelium [58,59].

In all cases, where increased estrogen levels were present either due to pellet implantation in OVX mice or naturally (pregnant or lactating animals), fenestrated vessels were observed in significantly greater samples compared to samples when estrogen levels were low ($-E_2$) or decreasing (involuting) (Table 1). To determine whether VEGF presence was correlated with the occurrence of fenestrated vessels, RT-PCR was used to qualitatively measure VEGF isoform levels in the mammary gland and tumors (Fig. 4). Estrogen alone was sufficient to significantly increase VEGF levels of each isoform. However, only isoforms 120 and 164 were upregulated in pregnant and lactating mammary glands. Similarly, tumors express the smaller isoforms in greater proportion to 188. Involuting mammary gland expresses less of each isoform compared to tissues with increased estrogen, similar to OVX $-E_2$ tissues. The results of vascular proliferation, VEGF expression, and vascular morphology are summarized in Table 2.

Discussion

We are dedicated to understanding the function of all the VEGF isoforms and receptors in developmental and pathological angiogenesis. We believe the mammary gland is an excellent model for studying the effects of VEGF in development and pathology, since it is developmentally immature in newborn animals and differentiates in easily separable stages during the life span of the animal. The study of VEGF in the mammary gland is especially important because the development and differentiation of the mammary gland can influence its susceptibility to carcinogenesis as the animal matures [60]. The prevalence of VEGF and VEGF receptor involvement in developmental angiogenesis in other settings makes it extremely likely that VEGF and its receptors play an important role in the vascular changes in all phases of mammary growth. In fact, the vasculature of the mammary gland during pregnancy and lactation possesses similar morphologies to the vasculature after exposure to VEGF [59,61]. It has been increasingly recognized that the host tissue microenvironment significantly affects tumor vascular morphology, physiology, and endothelial gene expression [62,63]. To this end, we undertook studies to determine VEGF isoform expression in the virgin, pregnant, lactating, and involuting mammary gland and compare the findings to mammary tumors.

In order to complete our Year 3 tasks, it was necessary to generate and characterize Abs against the VEGF receptors, Flk and Flt. We were somewhat successful, in that, we generated pAb to Flk which are significantly more sensitive than commercially available Abs which are not useful for immunohistochemistry. It is hoped that ongoing studies will be able to use our newly generated Abs for immunohistochemical localization of Flk-1 in tissues and possibly for immunoelectron microscopy.

We have also characterized vascular morphological changes in mammary tumors and in normal mammary tissues which led to surprising and exciting results. In short, our findings suggest that estrogen can modulate vascular endothelial morphology, inducing fenestrations and clustered, fused caveolae. We have previously demonstrated that these morphologies are directly induced by VEGF [58] and with others have shown that these modifications lead to increased vascular permeability [58,64]. The presence of endothelial fenestrae and

clustered caveolae are strongly correlated with estrogen up-regulation of VEGF in mammary tissues. We hypothesize that estrogen up-regulation of VEGF may be a normal physiological mechanism in mammary tissues to increase vascular permeability at times of increased proliferation and metabolism (pregnancy and lactation).

(7) CONCLUSIONS

It will be necessary to demonstrate that the endothelial modifications in response to estrogen are due specifically to VEGF up-regulation and not simply a coincidence. Studies which block VEGF activity in the presence of estrogen and result in no endothelial modification are warranted. We believe these studies are important to mammary tumor biology because they give insight into the complex regulation of the mammary gland and provide further evidence of the importance and activity of tumor-secreted VEGF on the surrounding mammary vasculature. Furthermore, our studies have characterized VEGF isoform expression in normal mammary and tumor tissues, demonstrating that only the shorter isoforms are prevalent.

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(9) Appendices

Figure Legends

1. ELISA test of anti-Flk and anti-Flt Ab against membrane or cytosolic proteins from MS1 endothelial cells. Commercially available Ab from Santa Cruz laboratories is included for comparison. Only the ant-Flk Ab demonstrates differential binding to membrane proteins where the VEGF receptor is expected.

2. Immunoblot using anti-Flk Ab (72a & 72b) against MS1 membranes. Ab from Santa Cruz laboratories (S.C.) are included for comparison. The Abs recognize a band at 205,000 Da, consitent with Flk migration. The Ab also did not recognize any bands in the cytosolic fractions (data not shown).

3. Electron micrographs of mammary glands from an ovariectomized mouse implanted with an Estradiol pellet [OVX+E2] (A, B), or implanted with a placebo pellet [OVX-E2] (C), a pregnant mouse (D), or a lactating mouse (E). Fenestrated endothelium is not seen in OVX-E2 glands, which are similar to virgin mammary gland vascular endothelium having numerous unclustered caveolae. Whereas, OVX+E2 mammary gland vascular endothelium has numerous fenestrae and fused, clustered caveolae, indicative of VEGF stimulated endothelium. Similarly, pregnant and lactating glands have vasculae endothelium with fenestrae and clustered caveolae.

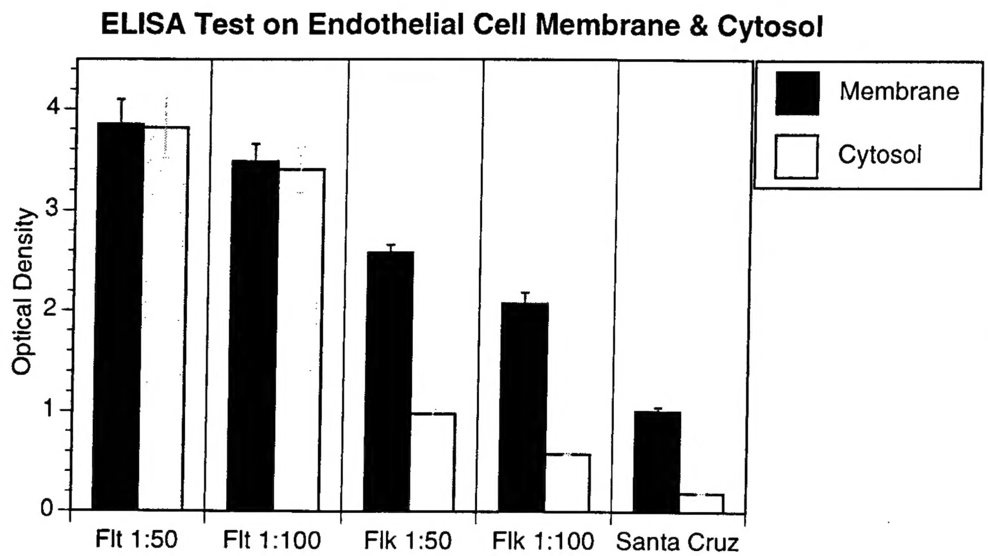
TABLE 1

	% samples with fenestrated endothelium
Mammary-E2	0 %
Mammary+E2	95%
Lactating	85%
Involuting	0%
Tumor	100%

Table 2

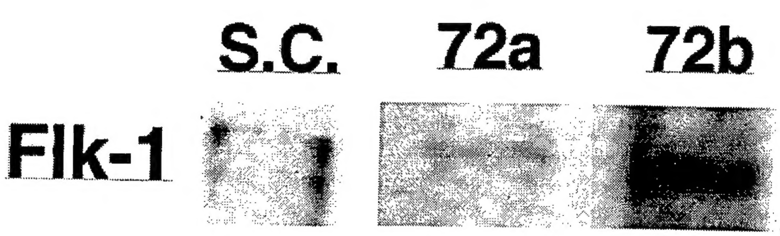
	Proliferative EC	VEGF expression	Fenestrated EC
Mammary-E2	-	-	-
Mammary+E2	+	++	++
Lactating	++	++	++
Pregnant	++	++	++
Involuting	-	-	-
Tumor	++++	++++	+++

Fig. 1



ELISA Test on Endothelial Cell Membrane & Cytosol
Optical Density
0
1
2
3
4
Flt 1:50
Flt 1:100
Flk 1:50
Flk 1:100
Santa Cruz
Membrane
Cytosol

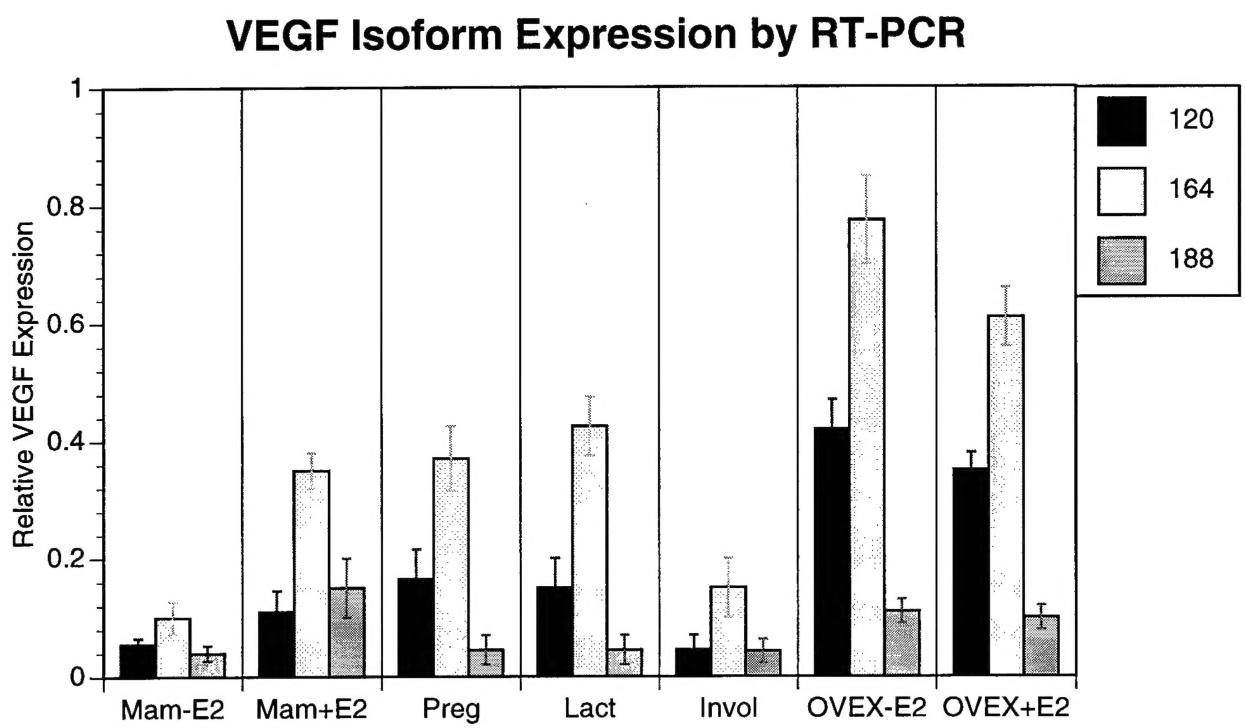
Fig. 2



S.C.
72a
72b
Flk-1

Figure 3

Fig. 4


VEGF Isoform Expression by RT-PCR
Relative VEGF Expression
0
0.2
0.4
0.6
0.8
1
Mam-E2
Mam+E2
Preg
Lact
Invol
OVEX-E2
OVEX+E2
120
164
188